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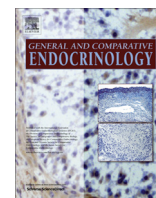
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# Testosterone influences song behaviour and social dominance – But independent of prenatal yolk testosterone exposure



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## ABSTRACT

In the last two decades, maternally derived yolk androgens have been shown to significantly alter offspring development, and a number of these effects persist into adulthood. However, little is known about their underlying mechanisms. Mechanisms that have been suggested are changes in the endogenous androgen production post-hatching or changes in the sensitivity towards circulating androgens.

We tested the effects of yolk testosterone on the plasma testosterone levels and the sensitivity to testosterone in 5 months old male canaries that hatched from eggs that were either injected with testosterone (yT-males) or with a control solution (yC-males). Changes in sensitivity were investigated via the behavioural response to an experimental elevation of the plasma testosterone levels. We performed the experiment in fall (low endogenous testosterone production), focusing on testosterone dependent response traits (aggression and song).

Before implantation, there was a non-significant trend that the plasma testosterone levels were lower in yT-males than in yC-males. Elevating the plasma testosterone concentrations increased aggressiveness, song bout length and similarity of repeated song elements (=consistency), with the latter likely being a consequence of testosterone-driven song crystallization. However, these effects were not different among yT- or yC-males in any of the parameters. Thus, our findings render it unlikely that changes in the sensitivity to testosterone post-hatching would form the main underlying mechanism of hormone-mediated maternal effects in birds. Further experiments are urgently needed in order to understand the nature of the phenotypic effects resulting from embryonic exposure to maternal yolk testosterone.

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## 1. Introduction

Hormones of maternal origin as found in the yolk of oviparous animals have been shown to significantly alter offspring phenotype (reviewed in Gil, 2003, 2008; Groothuis et al., 2005; von Engelhardt and Groothuis, 2011). In birds, the focus of research on maternally derived hormones has been on androgens, due to their often high abundance in the yolk and the existing knowledge of the potential of androgens to induce phenotypic changes (Groothuis and Schwabl, 2008). It is now commonly assumed that yolk androgens may provide a significant pathway along which the offspring phenotype can be altered in response to environmental conditions experienced by the mother. This view is based on (a) the fact that females differ in their yolk hormone allocation pattern in relation to a range of environmental circumstances and (b) the functional consequences for the offspring by affecting a large range of

different traits from early development up to adulthood (reviewed in Gil, 2003, 2008; Groothuis et al., 2005; von Engelhardt and Groothuis, 2011). However, we still know very little about the underlying mechanisms of maternal yolk hormone deposition and hormone-mediated maternal effects (Carere and Balthazart, 2007; Groothuis and Schwabl, 2008) – despite the wealth of behavioural and evolutionary ecological studies on (systematic) variation in yolk hormone levels and their consequences for offspring development. One pathway that has often been suggested is that yolk androgens alter the functioning of the hypothalamus–pituitary–gonadal (HPG) axis, leading to changes in sex steroid production. This has indeed been shown, but the directions of these effects varied: a non-significant increase following yolk T injections in quail chicks (Daisley et al., 2005), a significant increase in spotless starling chicks (Müller et al., 2007), a decrease in chicks of the domestic chicken (Pfannkuche et al., 2011), while there were no effects in chicken embryos (Pfannkuche et al., 2013) and adult house sparrows (Partecke and Schwabl, 2008) (see also Table 1).

An alteration of the HPG axis may also relate to a change in androgen receptor densities within the brain, which would lead to a different sensitivity towards androgen exposure post-hatching

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**Table 1**

Evidence for post-hatching changes in the endogenous testosterone production following experimental manipulation of the yolk androgen concentrations. Studies are sorted according to the age at plasma testosterone measurement. (abbreviations: T, testosterone; A<sub>4</sub>, androstenedione; mode, developmental mode; A, altricial; P, precocial).

Species	In ovo treatment	Age (days)	Mode	Sex difference	Effect
Domestic chicken ( <i>Gallus gallus domesticus</i> ) <sup>a</sup>	75 ng T	Embryo	P	♂ = ♀	No significant difference
Domestic chicken ( <i>Gallus gallus domesticus</i> ) <sup>b</sup>	75 ng T	14	P	♂ > ♀	Significant negative effect
Quail ( <i>Coturnix coturnix</i> ) <sup>c</sup>	50 ng T	14	P	♂ < ♀	Non significant positive trend
Spotless starling ( <i>Sturnus unicolor</i> ) <sup>d</sup>	5.6 ng T + 16.8 ng A <sub>4</sub>	15 or 17	A	♂ > ♀	Significant positive effect
Domestic chicken ( <i>Gallus gallus domesticus</i> ) <sup>e</sup>	75 ng T	154	P	♂ > ♀	No significant difference
Canary ( <i>Serinus canaria</i> ) <sup>f</sup>	50 ng T	270	A	n.a.	No significant difference
Canary ( <i>Serinus canaria</i> ) <sup>g</sup>	50 ng T	300	A	n.a.	Non significant negative trend
House sparrow ( <i>Passer domesticus</i> ) <sup>h</sup>	200 ng T	300–360	A	♂ > ♀	No significant difference

\* Females.

\* Males.

<sup>a</sup> Pfannkuche et al. (2013).

<sup>b</sup> Pfannkuche et al. (2011).

<sup>c</sup> Daisley et al. (2005).

<sup>d</sup> Müller et al. (2007).

<sup>e</sup> Riedstra et al. (2013).

<sup>f</sup> Müller et al. (2011).

<sup>g</sup> This study.

<sup>h</sup> Partecke and Schwabl (2008).

(sensu Groothuis and Schwabl, 2008). Indeed, Pfannkuche and colleagues (2011) showed not only a lower production of testosterone, but also a decrease in expression of androgen receptors in the brain of two week old chickens hatched from testosterone injected eggs, suggesting that the latter is the cause of the former via auto-regulation. However, a follow up study showed no changes in plasma testosterone, but again a decrease in androgen receptor density in chicken embryos at the end of the incubation period (Pfannkuche et al., 2013). The results suggest that these chicks have a lower sensitivity to plasma testosterone, in line with the effect of yolk testosterone feminizing competitive behaviour (Riedstra et al., 2013, see also Rubolini et al. 2006). However, in other species enhancing effects of yolk testosterone on androgen dependent behaviors/traits have been found (competitive behaviour: Eising et al., 2006; Müller et al., 2010; Partecke and Schwabl, 2008; Ruuskanen and Laaksonen, 2010; Schwabl, 1993; Strasser and Schwabl, 2004; nuptial plumage/secondary sexual traits Eising et al., 2006; Strasser and Schwabl, 2004; see also Müller and Eens, 2009). The latter suggests that yolk testosterone may increase the sensitivity to circulating plasma testosterone, since in some of these studies no increase in circulating plasma testosterone was found despite the fact that phenotypic changes were observed (e.g. Partecke and Schwabl, 2008). This increased sensitivity hypothesis is further supported by evidence provided by Ros et al. (2002), who found that early post-natal exposure to testosterone in gull chicks enhanced the sensitivity to a second testosterone treatment more than 30 days later – as reflected in their aggressive behaviour.

To test the effect of yolk testosterone on circulating plasma levels of testosterone and sensitivity to testosterone post-hatching, we established an experiment with male canaries (*Serinus canaria*), which hatched from eggs injected with either a testosterone solution or from eggs injected with a control solution only. At the age of five months, when natural testosterone levels are low (e.g. Nottebohm et al., 1987), we experimentally elevated their plasma testosterone concentrations and investigated the effects on two testosterone dependent behaviours. First, we focused on aggression, which has for a long time been linked to testosterone (e.g. Hau et al., 2000; Wingfield et al., 1987; Wingfield and Hahn, 1994; see also e.g. Soma et al., 2008 for a recent review and Sartor et al. 2005 for evidence in canaries). Furthermore, aggression has been shown to be influenced by yolk testosterone, among other in our study species (Eising et al., 2006; Müller et al. 2010; Partecke and Schwabl, 2008; Schwabl 1993; Strasser and Schwabl 2004; but see Müller et al., 2008; Ruuskanen and Laaksonen, 2010; Table 2).

Secondly, we looked at song expression, which is a trait known to vary with seasonal changes in testosterone concentrations (Catchpole and Slater, 2008; Nottebohm et al., 1987). We investigated the effects of the in ovo treatment and the subsequent testosterone implantation on song consistency, which reflects the temporal and spectral stereotypy of a song, because testosterone is thought to trigger the change from plastic, juvenile song, as sung in fall by first year canaries to consistent, stereotyped adult song, which is typically sung in spring after song crystallisation (Marler et al. 1988; Catchpole and Slater, 2008; Nottebohm, 2005; Williams, 2004). Song consistency has also been suggested to reflect a male's quality (Botero et al., 2009; de Kort et al., 2009; Sakata and Vehrencamp, 2011). Furthermore, a recent study in male canaries showed that implanting testosterone rapidly increased song activity (Sartor et al., 2005; Boseret et al., 2006). Thus, both song consistency and song activity likely form relevant song characteristics when studying changes in testosterone sensitivity – as function of embryonic exposure to experimentally manipulated yolk testosterone levels.

Given the previous evidence for long-lasting hormone-mediated maternal effects in our study species, we expected birds born from eggs with experimentally elevated yolk testosterone levels to show higher levels of aggressiveness, singing activity and song consistency prior to implantation, which may be linked to higher plasma testosterone levels. And we hypothesized that these birds would respond more sensitive to the testosterone implantation as reflected in a steeper increase in aggressive behaviour, singing activity and song consistency post-implantation.

## 2. Materials and methods

### 2.1. History of the experimental animals

This experiment was started in May 2011 under proper legislation of the Flemish and Belgian law and with approval by the animal experimentation committee of the University of Antwerp (number 2008-26). We selected 120 (60 male, 60 females) Fife Fancy canaries from our local breeding population. Each pair was placed in a separate cage (50 × 60 × 40 cm<sup>3</sup>, GEHU® cages) within the same room at a light cycle of 15:9 (L:D). We provided canary seed mixture (van Camp, Belgium), water shell grit, and cuttlefish bone ad libitum throughout the experiment. Egg food (van Camp, Belgium) was initially provided once a week, but daily as soon as chicks hatched. Egg laying was checked daily in the early morning

**Table 2**

Evidence for long-lasting effects of yolk androgens on aggressive behaviour or social dominance in a sexual context and/or during competition for food. (abbreviations: T, testosterone; A<sub>4</sub>, androstenedione; mode, developmental mode; A, altricial; P, precocial; SP, semi-precocial).

Species	In ovo treatment	Sex	Mode	Age (days)	Test context	Effect
Canary ( <i>Serinus canaria</i> ) <sup>a</sup>	correlative	Both	A	46–131	Food	Significant positive effect
Pied Flycatcher ( <i>Ficedula hypoleuca</i> ) <sup>b</sup>	7.5 ng T + 53.1 ng A <sub>4</sub>	Both	A	105	Food	No significant difference
Canary ( <i>Serinus canaria</i> ) <sup>c</sup>	50 ng T	Males	A	150	Sexual	No significant difference
House sparrow ( <i>Passer domesticus</i> ) <sup>d</sup>	200 ng T	Both	A	150	Food	Some significant positive effects
Domestic chicken ( <i>Gallus gallus domesticus</i> ) <sup>e</sup>	75 ng T	Both	P	154 + 180	Food	No significant difference
Canary ( <i>Serinus canaria</i> ) <sup>f</sup>	50 ng T	Males	A	180 + 360	Food	No significant difference
Canary ( <i>Serinus canaria</i> ) <sup>g</sup>	50 ng T	Both	A	210	Food	significant positive effect
Ring-necked pheasant ( <i>Phasianus colchicus</i> ) <sup>h</sup>	0.477 ng T <sup>*</sup>	Males	P	273	Food + sexual	No significant difference
Black-headed gull ( <i>Larus ridibundus</i> ) <sup>i</sup>	120 ng T + 10 µg A <sub>4</sub>	Both	SP	300	Food + sexual	Significant positive effect
House sparrow ( <i>Passer domesticus</i> ) <sup>j</sup>	200 ng T	Males	A	300–360	Sexual	Significant positive effect
House sparrow ( <i>Passer domesticus</i> ) <sup>j</sup>	200 ng T	Females	A	300–360	Sexual	No significant difference

<sup>\*</sup> Injected in albumen.

<sup>a</sup> Schwabl (1993).

<sup>b</sup> Ruuskanen & Laaksonen.

<sup>c</sup> This study.

<sup>d</sup> Strasser and Schwabl (2004).

<sup>e</sup> Riedstra et al. (2013).

<sup>f</sup> Müller et al. (2008).

<sup>g</sup> Müller et al. (2010).

<sup>h</sup> Bonisoli-Alquati et al. (2011).

<sup>i</sup> Eising et al. (2006).

<sup>j</sup> Partecke and Schwabl (2008).

and fresh eggs were weighed, marked and returned to the mother. The first two eggs were injected both with either a testosterone solution (50 ng testosterone/5 µL sesame oil) or a control solution (5 µL sesame oil) on the day the mother laid the third egg. Thus, all eggs within a clutch received the same treatment. The injected dose elevated the testosterone levels of the first-laid eggs to the levels of the last laid eggs [first/s egg:  $45.01 \pm 26.57$  ng/yolk,  $N = 14$ ; third/fourth egg:  $68.09 \pm 44.16$  ng/yolk,  $N = 11$ ; both mean  $\pm$  standard deviation, Müller et al., 2010]. We used a previously established injection protocol (Vergauwen et al., 2011). Briefly, eggs were cleaned before injecting the respective solution directly into the yolk of the egg. The hole in the eggshell at the injection site was closed with Opsite® (Smith and Nephew, Zaventem, Belgium), and the eggs were immediately returned to their nest. Only chicks hatching from the first- or second-laid egg were part of this study. The hatchability was similar for both treatments (80% of the control eggs and 75% of testosterone-treated eggs hatched, Pearson's chi square 0.52,  $p = 0.47$ ). Chicks were marked with a permanent marker for individual recognition at hatching, and ringed with a closed metal ring when  $\geq 7$  g. At independence (approximately 30 days old), the chicks were moved to indoor aviaries ( $2 \times 2 \times 2$  m<sup>3</sup>). All male offspring to be used in this experiment was housed in a single aviary together with adult tutor males. They were provided with canary seed mixture food and water ad libitum until the start of this experiment.

## 2.2. Experimental design

When 5 months old (=juveniles, not yet sexually mature, low endogenous testosterone levels), one male offspring per pair was selected for the experiment, resulting – due to hatching failure, chick mortality and female-only broods – in 24 unrelated males. Males were born from eggs injected with oil-only (control, referred to as yC-males,  $n = 15$ ) or oil containing testosterone (referred to as yT-males,  $n = 9$ ). These males were moved to a separated room and individually caged ( $50 \times 60 \times 40$  cm<sup>3</sup>). They were visually separated, but within auditory contact. Each of these cages was equipped with food and water, as well as with an omnidirectional tie clip microphone (TCM141 – AV-JEFE; frequency range: 30–18,000 Hz; impedance: 1000 Ω).

## 2.3. Behavioural tests

### 2.3.1. Song recordings

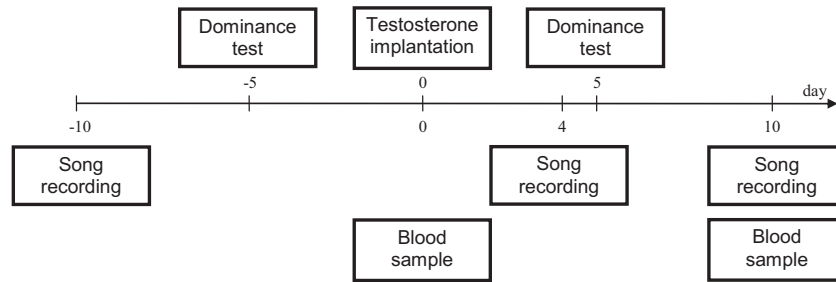
The microphone was connected with an M-audio MicroTrack 24–96 professional mobile digital recorder for song recordings. Birds were allowed to habituate to the new environment for 1 week before starting the song recordings. We recorded 2 h of song in the morning, as soon as the lights were switched on at three time points: 10 days before, 4 days after and 10 days after implantation (Fig. 1), given that the effects of testosterone implantation are established after one week (Heid et al., 1985).

### 2.3.2. Social dominance

We measured the social dominance of all birds five days before and five days after implantation (Fig. 1) following a previously established procedure (Müller et al., 2010). Briefly, all birds were weighed and we selected for each focal male a body mass matched same-age non-treated male from the local population (=opponent). All these opponents were placed in small individual cages ( $30 \times 40 \times 40$  cm<sup>3</sup>, GEHU®) within the same room. All birds, i.e. experimental birds and opponents, were subsequently food deprived for 18 h. We then performed staged encounters of the body mass matched opponent pairs in a cage that was equipped with two perches, a water bottle and one food container with limited seeds. We recorded their behaviour for 10 min with a Sony DCR SX30 camcorder for later behavioural analyses.

## 2.4. Hormone manipulation

All birds received a testosterone implant 10 days after the initial song recording (Fig. 1, day 0). We first obtained a blood sample (75 µL) by pricking the brachial vein of the birds with a 25 G needle and collected the blood sample in a Microvette® CB 300 (Sarstedt, Nümbrecht, Germany). Subsequently, we centrifuged the blood sample for 10 min at 3000 rpm, separated the plasma and stored it at  $-80$  °C freezer until analysis. Subsequently, we implanted all birds with closed Silastic tubes (Dow Corning, Midland, MI, USA No. 602–175; 0.76 mm inner diameter, 1.65 mm outer diameter) filled to 10 mm with crystalline testosterone (Sigma–Aldrich, Bornem, Belgium). The implant was inserted subcutaneously on top of



**Fig. 1.** A chronological representation of the experiment with respect to the day of implantation (=day 0). The first measurements were taken 10 days before implantation (=day –10) and the last measurements 10 days after implantation (=day 10). Text boxes describe the performed measurements/manipulations at the day indicated on the central time axis.

the pectoral muscle under local anaesthesia (Xylocaine, 10%) (see Sartor et al., 2005 for a similar methodology and dosage). 10 days post-implantation, a second blood sample was collected and the plasma was again stored at  $-80^{\circ}\text{C}$  for later hormone analysis.

## 2.5. Behavioural analysis

### 2.5.1. Song recordings

We analysed the first thirty minutes of each recording in Avisoft-SASLab Pro 5.2.05 (Avisoft Bioacoustics, Berlin, Germany; Sonogram parameters: FFT length 256, Frame Size 100% and overlap of 50%; filtered between 3 and 8 kHz to remove low and high frequency background noise, assuring no canary sang outside this frequency range). For each individual we counted the number of song bouts and measured their duration. In addition, we established a library of all the occurring syllable types and then selected a syllable type that was sung by all males (subsequently referred to as common syllable) (Fig. 2). The consistency of this particular syllable, thus the similarity of the syllable during a series of stereotypic repetitions within a song bout or between different song bouts, was measured. First the consistency of the common syllable across different song bouts (=between song bout consistency) was calculated by selecting the first common syllable per song bout, for 10 different song bouts. Secondly, we calculated the within song consistency as similarity of the common syllable during its

stereotypic repetition within the same song bout. To achieve this, we selected the first 15 times the common syllable was sung (=within song bout consistency). One bird did not repeat this common syllable within a song bout and was thus excluded for this particular analysis.

The consistency was calculated using Avisoft. The spectrograms were extracted and then uploaded in Avisoft-CORRELATOR version 2.2 2008. The spectrograms were subsequently compared using an analysis of spectrographic cross-correlation (SPCC), which has previously been shown to accurately reflect the structural syllable similarity (Clark et al., 1987; Rivera-Gutierrez et al., 2010). Song consistency can be assessed by means of spectrographic cross-correlations (SPCC) (see Rivera-Gutierrez et al., 2010 for a more detailed description of the procedure).

### 2.5.2. Social dominance

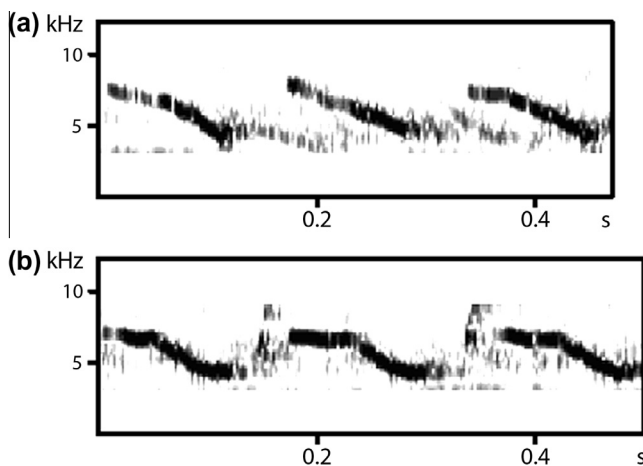
Videos were analysed using The observer XT 10.0 (Noldus, Wageningen, The Netherlands). We scored the number of wins (successful take-overs of the food source or winning overt fights) and the time spent at the feeder as a measure for dominance. These values were corrected for the among staged encounter differences in fighting intensity. To achieve this, we divided the number of wins of the focal bird by the total number of fights during the recorded period. Similarly, we divided the time spent at the feeder by the focal bird by the total time both birds spent at the feeder. There was no singing activity during the staged encounters, implying that this behaviour could not be studied.

## 2.6. Hormone analysis

Testosterone concentrations were measured following a previously established method (Goerlich et al., 2009). Briefly, the plasma was thawed and 2.5 mL of a 70:30 (vol/vol) diethylether/petroleum benzine solution was added. The tubes were vortexed, centrifuged for 4 min at 13,000 rpm, snap frozen and decanted. The extract was dried under a nitrogen stream. The same procedure was repeated once, followed by a single extraction with 1 mL methanol (70%) and the samples were then stored over night at  $-20^{\circ}\text{C}$ . The day after, the methanol phase was decanted and again dried under a nitrogen stream. The sample was re-suspended in 80  $\mu\text{L}$  PBS-G. 50  $\mu\text{L}$  of this suspension was used in one RIA. We used a commercially available RIA kit (Spectria® Testosterone RIA, Orion Diagnostica; detection limit 0.03 ng/mL with; cross-reactivity 100% with testosterone, 0.03% with androstenedione and 4.5% with DHT). The intra-assay cross variance was 7.7%.

## 2.7. Statistical analysis

Statistical analyses were performed in SPSS 20.0. All data were checked for normal distribution and equality of the variances and



**Fig. 2.** Three repetitions of the common syllable type, which was sung by all males, within the song of one experimental male. Syllables differed more before implantation reflecting typical plastic juvenile song (a), while their similarity increased after implantation due to song crystallization (b). Indicated by small differences in structural similarity in syllable shape especially at the start of each syllable. The song recording was filtered between 3 and 8 kHz, to remove low and high frequency background noise. Black lines reflect the common syllable, while lighter grey lines reflect background noise, which fell within the same frequency of the common syllable.



transformations applied if necessary. Potential long-lasting effects of experimentally elevated yolk testosterone levels on song, social dominance and plasma testosterone were tested by comparing the pre-implantation values between yT- and yC-males using independent samples *t*-tests. The effect of the implant and the interaction with the in ovo treatment was analysed using a repeated measures ANOVA, with day of measurement as repeated measure (within individuals) and in ovo treatment as fixed effect (among individuals). Results were considered significant at the  $\alpha \leq 0.05$  level. Body mass was initially included as covariate, but never contributed significantly to the models (all  $p > 0.55$ ) and, therefore, removed from the final models. These results are not presented.

### 3. Results

#### 3.1. Initial differences between yT- and yC-males prior to implantation

Males that hatched from eggs with experimentally elevated yolk testosterone levels (yT-males,  $n = 9$ ) tended to have lower plasma testosterone concentrations than males that hatched from control eggs (yC-males,  $n = 15$ ) at the day of implantation (treatment:  $t_{15,22} = 2.08$ ;  $p = 0.05$ ) (Fig. 3). The number of wins and the time spent at the feeder did not differ between yT- and yC-males 5 days before implantation (relative number of wins:  $t_{22} = -0.34$ ;  $p = 0.74$ ; relative time spent at feeder:  $t_{22} = 1.02$ ;  $p = 0.32$ ; Cohen's

$d = 0.43$ ) (Fig. 4a,b). The in ovo testosterone treatment did not affect song consistency within the same song bout ( $t_{21} = 1.45$ ;  $p = 0.16$ ) (Fig. 5a), song consistency between song bouts ( $t_{22} = 1.29$ ;  $p = 0.21$ ) (Fig. 5b), the mean song bout length ( $t_{22} = 1.21$ ;  $p = 0.24$ ) (Fig. 6a) or the number of song bouts ( $t_{22} = 1.00$ ;  $p = 0.33$ ) (Fig. 6b), all measured 10 days before implantation when the males were about 5 months old.

#### 3.2. Consequences of implantation for yT- and yC-males

##### 3.2.1. Hormone concentration

The implant significantly increased the plasma testosterone concentrations ( $F_{1,22} = 96.47$ ;  $p < 0.001$ ), but this increase did not differ between yT- and yC-males (in ovo treatment  $\times$  time:  $F_{1,22} = 0.34$ ;  $p = 0.56$ ) (Fig. 3). There was no overall difference in the plasma testosterone concentration between yT- and yC-males ( $F_{1,22} = 0.002$ ;  $p = 0.97$ ).

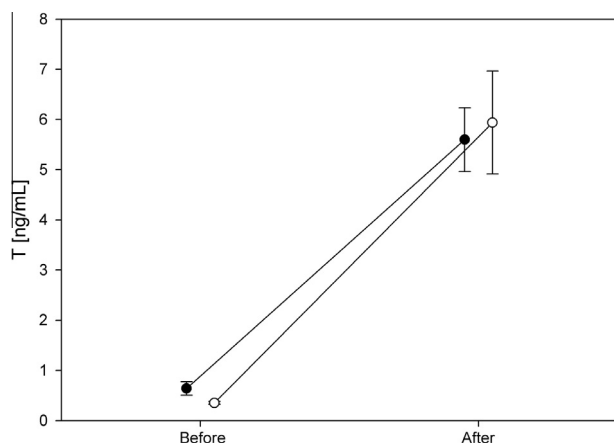
##### 3.2.2. Social dominance

There was a significant increase in the relative number of wins following implantation ( $F_{1,22} = 7.92$ ;  $p = 0.01$ ) and a non-significant statistical trend in the same direction for the time spent at the feeder ( $F_{1,22} = 3.94$ ;  $p = 0.06$ ). However, in neither of the two traits did the effect of implantation differ between yT- and yC-males (relative number of wins: in ovo treatment  $\times$  time:  $F_{1,22} = 0.78$ ;  $p = 0.39$ ; time spent at the feeder: in ovo treatment  $\times$  time:  $F_{1,22} = 1.72$ ;  $p = 0.20$ ). In both cases there was no overall effect of in ovo treatment (relative number of wins:  $F_{1,22} = 0.68$ ;  $p = 0.42$ ; time spent at the feeder:  $F_{1,22} = 0.22$ ;  $p = 0.65$ ) (Fig. 4a and b).

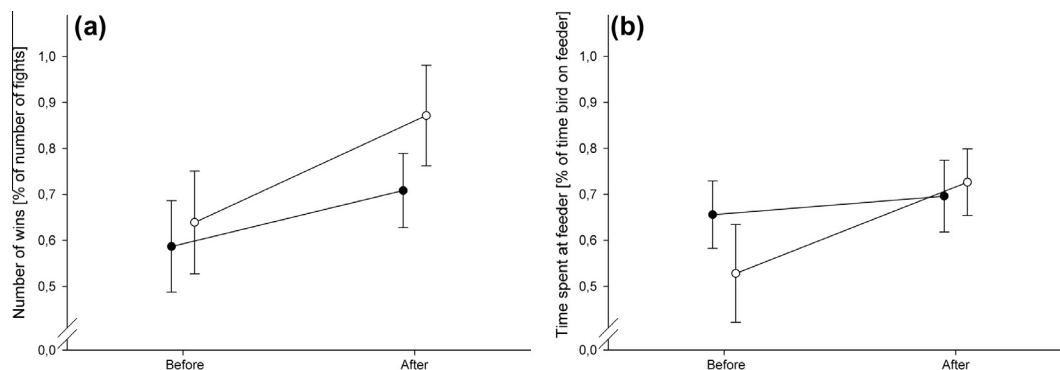
##### 3.2.3. Song

There was a significant increase in song consistency both within and between song bouts after implantation (within song bouts:  $F_{2,38} = 16.94$ ;  $p < 0.001$ ; between song bouts:  $F_{2,38} = 15.92$ ;  $p < 0.001$ ) (Fig. 5a and b). This change in song consistency with implantation was not influenced by the in ovo treatment (within song bouts: in ovo treatment  $\times$  time:  $F_{2,38} = 1.56$ ;  $p = 0.22$ ; between song bouts: in ovo treatment  $\times$  time:  $F_{2,38} = 0.48$ ;  $p = 0.63$ ). There was no general difference between yC- and yT-males in their within song bout consistency ( $F_{1,19} = 1.39$ ;  $p = 0.25$ ), but a non-significant trend that yC-males had a higher between song bout consistency than yT-males ( $F_{1,19} = 4.08$ ;  $p = 0.06$ ) (Fig. 5a and b).

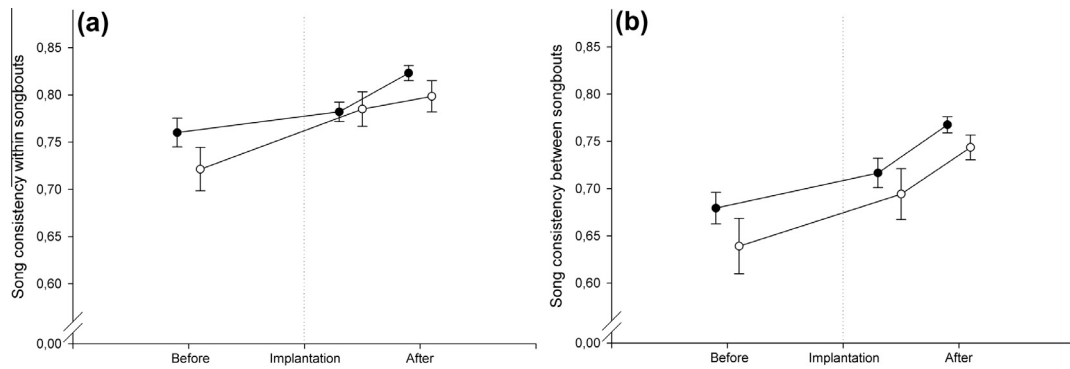
The average song bout duration significantly increased after implantation ( $F_{1,60,35,10} = 13.50$ ;  $p < 0.001$ ), but the effect of implantation was again not influenced by the in ovo treatment (in ovo treatment  $\times$  time:  $F_{1,60,35,10} = 2.49$ ;  $p = 0.11$ ). There was no



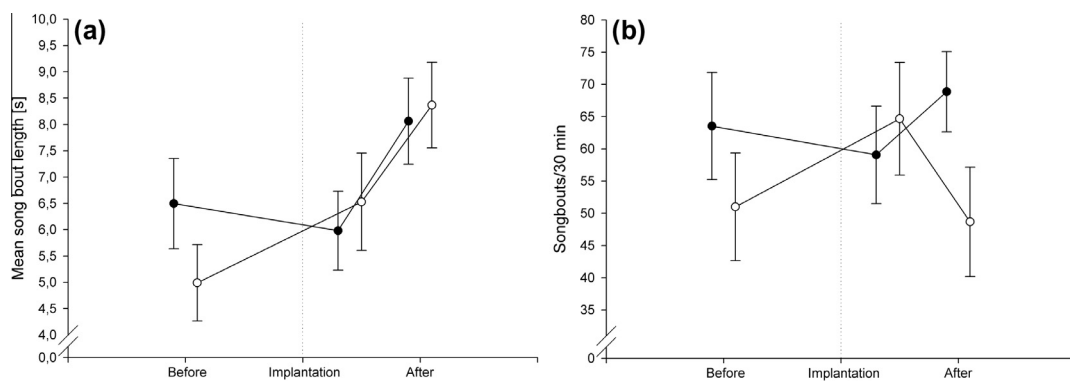
**Fig. 3.** Testosterone concentrations significantly increased after implantation in both males born from control eggs (yC-males, filled circles) and males born from testosterone treated eggs (yT-males, open circles). Values per group are separated for visualisation only.



**Fig. 4.** The total number of wins increased post-implantation, but did not vary significantly between birds hatched from control eggs (yC-males, filled circles) and birds born from testosterone treated eggs (yT-males, open circles) (a). The total time birds spent at the feeder tended to be higher after implantation, but was not different between yC- or yT-males (b).



**Fig. 5.** The experimental elevation of the plasma testosterone levels increased the song consistency within (a) and among (b) song bouts. However, this effect did not vary with the in ovo testosterone treatment, nor was there an overall difference between birds born from control eggs (yC-males, filled circles) and birds born from testosterone eggs (yT-males, open circles). The dotted line indicates the moment of implantation.



**Fig. 6.** The mean song bout duration (a) but not the number of song bouts (b) increased significantly after testosterone implantation. However, the observed effects were independent from the fact whether a birds was born from control eggs (yC-males, filled circles) or from testosterone treated eggs (yT-males, open circles). The dotted line indicates the moment of implantation, values per group are scattered to improve the readability of the graph.

effect of experimentally elevated yolk testosterone levels on the average song bout length ( $F_{1,22} = 0.04$ ;  $p = 0.84$ ) (Fig. 6a).

The number of song bouts in the recording period (=song rate) was not significantly affected by the implantation ( $F_{1,23,27,00} = 0.28$ ;  $p = 0.65$ ). There was also no indication that the implantation affected yC-males more than yT-males or vice versa (in ovo treatment  $\times$  time:  $F_{1,23,27,00} = 2.27$ ;  $p = 0.14$ ), neither did yC-males differ from yT-males ( $F_{1,22} = 0.97$ ;  $p = 0.33$ ) (Fig. 6b).

#### 4. Discussion

The mechanisms underlying yolk hormone-mediated maternal effects remain – despite the wealth of behavioural and evolutionary ecological studies that have recently dealt with hormone-mediated maternal effects – largely unclear. Two mechanisms have frequently been suggested to mediate the short- and long-term phenotypic consequences that have been described, both relating to differences in the functioning of the hypothalamus–pituitary–gonadal (HPG) axis: changes in the endogenous androgen production or changes in the sensitivity towards circulating androgens e.g. due to altered receptor densities. Here we tested both potential mechanisms in juvenile canaries (5 months old) that hatched from eggs with experimentally manipulated yolk testosterone levels by experimentally elevating the plasma testosterone levels of juvenile canaries. We show based on a number of behavioural measures that there is no evidence of a long-lasting change in sensitivity towards testosterone following embryonic exposure to maternal testosterone.

##### 4.1. Initial differences between yT- and yC-males prior to testosterone implantation

There were no significant differences in song behaviour or social dominance prior to the experimental elevation of the plasma testosterone concentrations. The latter is contrasting earlier studies showing a positive effect of elevated yolk androgens on social dominance (Table 2). However, most of these studies tested for differences in competitiveness in a treatment paired design, which may overestimate the actual effect, because winning by the treated male by definition implies losing of a control-bird. In addition, here dyads have been matched for body mass, which may mask effects that result from yolk testosterone stimulated offspring growth and thus adult size (e.g. in canaries: Müller et al., 2010; Schwabl, 1996; Vergauwen et al., 2011). However, it remains as yet unclear why the effects on social dominance tend to differ among studies and study species.

Prior to implantation, yT-males tended to have lower plasma testosterone levels than yC-males, but this was not statistically significant. Lowered plasma testosterone concentrations following an in ovo manipulation of the yolk testosterone levels have been reported for two week old chickens (Pfannkuche et al., 2011), while two previous studies in quail respectively spotless starlings reported rather increased testosterone production post-hatching (Daisley et al., 2005; Müller et al., 2007). No differences were found in chicken embryos (Pfannkuche et al., 2013) and in adult house sparrows measured in their first spring (Partecke and Schwabl, 2008), when testosterone levels are typically elevated. Thus, the evidence for the effect of yolk testosterone on plasma testosterone

levels post-hatching is still ambiguous (Table 1). There is no clear pattern in relation to potential factors such as developmental mode, domestication, injected dose, or life stage. The latter may in fact alter the effects of yolk testosterone due to a rise in endogenous production, changes in the source of testosterone production, or changes in the role of testosterone in regulating a given behaviour. However, thus far no study measured plasma testosterone levels at more than one life stage for the same individuals, which would certainly form a fruitful avenue for further research, and the number of studies is still comparatively low. Finally, the effects of prenatal exposure to testosterone may differ between sexes (Pfannkuche et al., 2011), which cannot be further addressed here as only males were tested.

#### 4.2. Consequences of testosterone implantation

There was a significant increase in the plasma testosterone concentration, measured 10 days post implantation. The plasma testosterone concentrations were within the physiological range of what has previously been measured in our population during the breeding season (post-implantation:  $5.73 \pm 0.54$  ng/mL, breeding season:  $4.94 \pm 0.46$  ng/mL, unpublished data). The increase in plasma testosterone concentrations was accompanied by an increase in all but one response traits that were investigated.

The implant led to an increase in aggressive behaviour during a staged encounter and tended to increase the success in defending the food source. The activating effect of testosterone on aggression has extensively been shown in temperate zone bird species (e.g. Hau et al., 2000; Wingfield and Hahn, 1994; but see Sartor et al., 2005). Although the role of testosterone in eliciting aggressive responses can be context dependent, as it may vary with time of the year, or between species according to differences in life-history, as e.g. the degree of territoriality throughout the year (Hau, 2007; Soma, 2006). In canaries, agonistic behaviour may represent an example for a testosterone-enhanced behaviour given that it occurs outside the breeding season (here: prior to implantation), as in many other songbirds when the plasma testosterone levels are low (e.g. Schwabl and Kriner, 1991; Soma et al., 2002), while testosterone increases its intensity and probability when present.

Testosterone has also been shown to trigger important aspects of bird song, among others song crystallization (Marler et al., 1988; Korsia and Bottjer, 1991; Templeton et al., 2012). The stereotypy of song has been shown to increase in spring, when testosterone levels are high compared to fall, when testosterone levels are low (Nottebohm et al., 1986; Smith et al., 1997; but see Van Hout et al., 2012). Thus, the increase in song consistency both within and between songs as observed post-implantation most likely reflects testosterone-driven song crystallization.

When interpreting the results, it has to be considered that not all aspects of the song system were fully mature at 5 months of age, even though all important brain areas are sexually dimorphic and have reached adult size already at one month of age (Alvarez-Buylla et al., 1988, Gahr et al., 1996). However, stable adult song is reached between 6–10 months of age (Nottebohm et al., 1986), suggesting that some maturation of the song system may be achieved only by this age. Our manipulation was, therefore, likely done during ongoing dynamic developmental processes (Alvarez-Buylla et al., 1988), which may – similar to seasonal differences (e.g. Devoogd, 1991; Fusani et al., 2000; Voorhuis et al., 1991) – modulate the effects of testosterone. However, these developmental processes were apparently not systematically affected by the embryonic testosterone exposure, given the lack of a statistical difference in the traits investigated.

Testosterone implantation increased singing activity as expected, given that it has been shown to vary with the plasma testosterone levels in starlings and canaries (e.g. Absil et al., 2003;

Boseret et al., 2006; De Ridder et al., 2000; Pinxten et al., 2002; Sartor et al., 2005; Van Hout et al., 2012; but see Van Hout et al., 2009). Interestingly, this was not due to an increase in the number of songs, but rather to an increase in song duration. This last finding is in line with the increase in song bout length as typically seen in springtime, when hormone levels rise (Riters et al., 2001; Smith et al., 1997; Voigt and Leitner, 2008).

We did not find evidence that birds born from testosterone-treated eggs significantly differed in their response to an experimental elevation of the plasma testosterone concentrations from birds that hatched from control-treated eggs. yT- and yC-males did not differ in any of the behavioral traits investigated, although nearly all of these traits responded to the increase in plasma testosterone levels following implantation. Even if yT- and yC-males do not differ at a final time point, they may differ in their response curve in terms of the timing or concentrations of response to elevated plasma testosterone levels. However, we do not have evidence for such temporal differences as can be derived from the song traits that were measured at two different moments.

In conclusion, we did not find long-lasting phenotypic effects following an experimental yolk testosterone manipulation for the traits investigated and we did not find evidence for changes in the responsiveness to testosterone. Further studies measuring the effect of elevation of yolk testosterone on behaviour, circulating hormone levels and androgen receptor densities in the same individual and in different species should be conducted to reveal the underlying mechanisms of the phenotypic effects of yolk testosterone. It may also be relevant to consider additional pathways that may e.g. involve aromatase, the enzyme converting testosterone to estradiol. Aromatase activity can be regulated by among other testosterone (Balthazart and Ball, 1998), while it modulates the expression of a number of social and sexual behaviour including the activation of song and the seasonal neuroplasticity of the song system (Balthazart et al., 2010; Fusani and Gahr, 2006).

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#### References

- Absil, P., Pinxten, R., Balthazart, J., Eens, M., 2003. Effect of age and testosterone on autumnal neurogenesis in male European starlings (*Sturnus vulgaris*). *Behav. Brain Res.* 143, 15–30.
- Alvarez-Buylla, A., Theelen, M., Nottebohm, F., 1988. Birth of projection neurons in the higher vocal center of the canary forebrain before, during, and after song learning. *Proc. Natl. Acad. Sci.* 85, 8722–8726.
- Balthazart, J., Charlier, T.D., Barker, J.M., Yamamura, T., Ball, G.F., 2010. Sex steroid-induced neuroplasticity and behavioral activation in birds. *Eur. J. Neurosci.* 32, 2116–2132.
- Balthazart, J., Ball, G.F., 1998. New insights into the regulation and function of brain estrogen synthase (aromatase). *Trend Neurosci.* 21, 243–249.
- Bonisoli-Alquati, A., Matteo, A., Ambrosini, R., Rubolini, D., Romano, M., Caprioli, M., Dessi-Fulgheri, F., Baratti, M., Saino, N., 2011. Effects of egg testosterone on female mate choice and male sexual behavior in the pheasant. *Horm. Behav.* 59, 75–82.
- Boseret, G., Carere, C., Ball, G.F., Balthazart, J., 2006. Social context affects testosterone-induced singing and the volume of song control nuclei in male domestic canaries (*Serinus canarius*). *J. Neurobiol.* 66, 1044–1060.
- Botero, C.A., Rossman, R.J., Caro, L.M., Stenzler, L.M., Lovette, I.J., de Kort, S.R., Vehrencamp, S.L., 2009. Syllable type consistency is related to age, social status



- and reproductive success in the tropical mockingbird. *Anim. Behav.* 77, 701–706.
- Carere, C., Balthazart, J., 2007. Sexual versus individual differentiation: the controversial role of avian maternal hormones. *Trends Endocrinol. Metab.* 18, 73–80.
- Catchpole, C.K., Slater, P.J.B., 2008. *Bird Song*. 2nd ed. University Press, Cambridge.
- Clark, C.W., Marler, P., Beeman, K., 1987. Quantitative-analysis of animal vocal phonology—an application to swamp sparrow song. *Ethology* 76, 101–115.
- Daisley, J.N., Bromundt, V., Mostl, E., Kotrschal, K., 2005. Enhanced yolk testosterone influences behavioral phenotype independent of sex in Japanese quail chicks *Coturnix japonica*. *Horm. Behav.* 47, 185–194.
- de Kort, S.R., Eldermire, E.R.B., Valderrama, S., Botero, C.A., Vehrencamp, S.L., 2009. Trill consistency is an age-related assessment signal in banded wrens. *Proc. R. Soc. B Biol. Sci.* 276, 2315–2321.
- De Ridder, E., Pinxten, R., Eens, M., 2000. Experimental evidence of a testosterone-induced shift from paternal to mating behaviour in a facultatively polygynous songbird. *Behav. Ecol. Sociobiol.* 49, 24–30.
- Devoogd, T.J., 1991. Endocrine modulation of the development and adult function of the avian song system. *Psychoneuroendocrinology* 16, 41–66.
- Eising, C.M., Müller, W., Groothuis, T.G.G., 2006. Avian mothers create different phenotypes by hormone deposition in their eggs. *Biol. Lett.* 2, 20–22.
- Fusani, L., Van't Hof, T., Hutchison, J.B., Gahr, M., 2000. Seasonal expression of androgen receptors, estrogen receptors, and aromatase in the canary brain in relation to circulating androgens and estrogens. *J. Neurobiol.* 43, 254–268.
- Fusani, L., Gahr, M., 2006. Hormonal influence on song structure and organization: the role of estrogen. *Neuroscience* 138, 939–946.
- Gil, D., 2003. Golden eggs: maternal manipulation of offspring phenotype by egg androgens in birds. *Ardeola* 50, 281–294.
- Gil, D., 2008. Hormones in avian eggs: Physiology, ecology and behavior. In: Brockmann, H.J., Roper, T.J., Naguib, M., Wynne-Edwards, K.E., Barnard, C., Mitani, J.C. (Eds.), *Advances in the Study of Behavior*, 38. Elsevier Academic Press Inc, San Diego, pp. 337–398.
- Goerlich, V.C., Dijkstra, C., Schaafsma, S.M., Groothuis, T.G.G., 2009. Testosterone has a long-term effect on primary sex ratio of first eggs in pigeons-in search of a mechanism. *Gen. Comp. Endocrinol.* 163, 184–192.
- Groothuis, T.G.G., Müller, W., von Engelhardt, N., Carere, C., Eising, C., 2005. Maternal hormones as a tool to adjust offspring phenotype in avian species. *Neurosci. Biobehav. Rev.* 29, 329–352.
- Groothuis, T.G.G., Schwabl, H., 2008. Hormone-mediated maternal effects in birds: mechanisms matter but what do we know of them? *Philos. Trans. R. Soc. B Biol. Sci.* 363, 1647–1661.
- Hau, M., 2007. Regulation of male traits by testosterone: implications for the evolution of vertebrate life histories. *Bioessays* 29, 133–144.
- Hau, M., Wikelski, M., Soma, K.K., Wingfield, J.C., 2000. Testosterone and year-round territorial aggression in a tropical bird. *Gen. Comp. Endocrinol.* 117, 20–33.
- Heid, P., Göttinger, H.-R., Pröve, E., 1985. The influence of castration and testosterone replacement on the song architecture of canaries (*Serinus canaria*). *Z. Tierpsychol.* 69, 224–236.
- Korsia, S., Bottjer, S.W., 1991. Chronic testosterone treatment impairs vocal learning in male zebra finches during a restricted period of development. *J. Neurosci.* 11, 2362–2371.
- Marler, P., Peters, S., Ball, G.F., Dufty, A.M., Wingfield, J.C., 1988. The role of sex steroids in the acquisition and production of birdsong. *Nature* 336, 770–772.
- Müller, W., Boonen, S., Groothuis, T.G.G., Eens, M., 2010. Maternal yolk testosterone in canary eggs: towards a better understanding of mechanism and function. *Behav. Ecol.* 21, 493–500.
- Müller, W., Deptuch, K., Lopez-Rull, I., Gil, D., 2007. Elevated yolk androgen levels benefit offspring development in a between-clutch context. *Behav. Ecol.* 18, 929–936.
- Müller, W., Eens, M., 2009. Elevated yolk androgen levels and the expression of multiple sexually selected male characters. *Horm. Behav.* 55, 175–181.
- Müller, W., Vergauwen, J., Eens, M., 2008. Yolk testosterone, postnatal growth and song in male canaries. *Horm. Behav.* 54, 125–133.
- Müller, W., Groothuis, T.G.G., Goerlich, V.C., Eens, M., 2011. GnRH - a missing link between testosterone concentrations in yolk and plasma and its intergenerational effects. *PLoS One* 6, e22675.
- Nottebohm, F., 2005. The neural basis of birdsong. *PLoS Biol.* 3, 759–761.
- Nottebohm, F., Nottebohm, M.E., Crane, L., 1986. Developmental and seasonal changes in canary song and their relation to changes in the anatomy of song control nuclei. *Behav. Neural Biol.* 46, 445–471.
- Nottebohm, F., Nottebohm, M.E., Crane, L.A., Wingfield, J.C., 1987. Seasonal-changes in gonadal hormone levels of adult male canaries and their relation to song. *Behav. Neural Biol.* 47, 197–211.
- Pardecke, J., Schwabl, H., 2008. Organizational effects of maternal testosterone on reproductive behavior of adult house sparrows. *Dev. Neurobiol.* 68, 1538–1548.
- Pfannkuche, K.A., Gahr, M., Weites, I.M., Riedstra, B., Wolf, C., Groothuis, T.G.G., 2011. Examining a pathway for hormone mediated maternal effects - Yolk testosterone affects androgen receptor expression and endogenous testosterone production in young chicks (*Gallus gallus domesticus*). *Gen. Comp. Endocrinol.* 172, 487–493.
- Pfannkuche, K.A., Gahr, M., Weites, I.M., Riedstra, B., Wolf, C., Groothuis, T.G.G., 2013. Examining a pathway for hormone mediated maternal effects - yolk testosterone decreases androgen receptor mRNA expression sex specifically and independently of circulating testosterone concentrations in chicken embryos. In: Pfannkuche, K.A. (Ed.), *A testosterone's tale - exploring different pathways for testosterone mediated maternal effects* (Ph.D. thesis). Rijksuniversiteit Groningen, Groningen, The Netherlands, pp. 168–189.
- Pinxten, R., De Ridder, E., Balthazart, J., Eens, M., 2002. Context-dependent effects of castration and testosterone treatment on song in male European starlings. *Horm. Behav.* 42, 307–318.
- Riedstra, B., Pfannkuche, K.A., Groothuis, T.G.G., 2013. Increased exposure to yolk testosterone has feminizing effects in chickens (*Gallus gallus domesticus*). *Anim. Behav.* 85, 701–708.
- Riters, L.V., Baillien, M., Eens, M., Pinxten, R., Foidart, A., Ball, G.F., Balthazart, J., 2001. Seasonal variation in androgen-metabolizing enzymes in the diencephalon and telencephalon of the male European starling (*Sturnus vulgaris*). *J. Neuroendocrinol.* 13, 985–997.
- Rivera-Gutiérrez, H.F., Pinxten, R., Eens, M., 2010. Multiple signals for multiple messages: great tit, Parus major, song signals age and survival. *Anim. Behav.* 80, 451–459.
- Ros, A.F.H., Dieleman, S.J., Groothuis, T.G.G., 2002. Social stimuli, testosterone, and aggression in gull chicks: Support for the challenge hypothesis. *Horm. Behav.* 41, 334–342.
- Rubolini, D., Romano, M., Martinelli, R., Leoni, B., Saino, N., 2006. Effects of prenatal yolk androgens on armaments and ornaments of the ring-necked pheasant. *Behav. Ecol. Sociobiol.* 59, 549–560.
- Ruuskanen, S., Laaksonen, T., 2010. Yolk hormones have sex-specific long-term effects on behavior in the pied flycatcher (*Ficedula hypoleuca*). *Horm. Behav.* 57, 119–127.
- Sakata, J.T., Vehrencamp, S.L., 2011. Integrating perspectives on vocal performance and consistency. *J. Exp. Biol.* 215, 201–209.
- Sartor, J.J., Balthazart, J., Ball, G.F., 2005. Coordinated and dissociated effects of testosterone on singing behavior and song control nuclei in canaries (*Serinus canaria*). *Horm. Behav.* 47, 467–476.
- Schwabl, H., 1993. Yolk is a source of maternal testosterone for developing birds. *Proc. Natl. Acad. Sci. USA* 90, 11446–11450.
- Schwabl, H., 1996. Maternal testosterone in the avian egg enhances postnatal growth. *Comp. Biochem. Physiol. A Physiol.* 114, 271–276.
- Schwabl, H., Kriner, E.E., 1991. Territorial aggression and song of male European robins (*Erithacus rubecula*) in autumn and spring: effects of antiandrogen treatment. *Horm. Behav.* 25, 180–194.
- Smith, G.T., Brenowitz, E.A., Beecher, M.D., Wingfield, J.C., 1997. Seasonal changes in testosterone, neural attributes of song control nuclei, and song structure in wild songbirds. *J. Neurosci.* 17, 6001–6010.
- Soma, K.K., 2006. Testosterone and aggression: berthold, birds and beyond. *J. Neuroendocrinol.* 18, 543–551.
- Soma, K.K., Wissman, A.M., Brenowitz, E.A., Wingfield, J.C., 2002. Dehydroepiandrosterone (DHEA) increases territorial song and the size of an associated brain region in a male songbird. *Horm. Behav.* 41, 203–212.
- Soma, K.K., Scotti, M.A.L., Newman, A.E.M., Charlier, T.D., Demas, G.E., 2008. Novel mechanisms for neuroendocrine regulation of aggression. *Front. Neuroendocrinol.* 29, 476–489.
- Strasser, R., Schwabl, H., 2004. Yolk testosterone organizes behavior and male plumage coloration in house sparrows (*Passer domesticus*). *Behav. Ecol. Sociobiol.* 56, 491–497.
- Templeton, C.N., Burt, J.M., Campbell, S.E., Lent, K., Brenowitz, E.A., Beecher, M.D., 2012. Immediate and long-term effects of testosterone on song plasticity and learning in juvenile song sparrows. *Behav. Processes* 90, 254–260.
- Van Hout, A.J.M., Eens, M., Balthazart, J., Pinxten, R., 2009. Complex modulation of singing behavior by testosterone in an open-ended learner, the European starling. *Horm. Behav.* 56, 564–573.
- Van Hout, A.J.M., Pinxten, R., Darras, V.M., Eens, M., 2012. Testosterone increases repertoire size in an open-ended learner: an experimental study using adult male European starlings (*Sturnus vulgaris*). *Horm. Behav.* 62, 563–568.
- Vergauwen, J., Heylen, D., Eens, M., Müller, W., 2011. Negative effects of yolk testosterone and ticks on growth in canaries. *J. Exp. Zool. Part A* 315A, 553–561.
- Voigt, C., Leitner, S., 2008. Seasonality in song behaviour revisited: seasonal and annual variants and invariants in the song of the domesticated canary (*Serinus canaria*). *Horm. Behav.* 54, 373–378.
- von Engelhardt, N., Groothuis, T.G.G., 2011. Maternal hormones in avian eggs. In: Norris, D.O., Lopez, K.H. (Eds.), *Hormones and Reproduction of Vertebrates*, 4. Birds, Academic Press, pp. 91–127.
- Voorhuis, T.A.M., De Kloet, E.R., De Wied, D., 1991. Effect of a vasotocin analog on singing behavior in the canary. *Horm. Behav.* 25, 549–559.
- Williams, H., 2004. Birdsong and singing behavior. In: Zeigler, H.P., Marler, P. (Eds.), *Behavioral Neurobiology of Birdsong*. New York Acad Sciences, New York, pp. 1–30.
- Wingfield, J.C., Ball, G.F., Dufty, A.M., Hegner, R.E., Ramenofsky, M., 1987. Testosterone and aggression in birds. *Am. Scientist* 75, 602–608.
- Wingfield, J.C., Hahn, T.P., 1994. Testosterone and territorial behavior in sedentary and migratory sparrows. *Anim. Behav.* 47, 77–89.